ALPHA INTERFERON VARIANTS

FIELD OF THE INVENTION

The present invention relates to biologically active variants of human alpha interferon.

BACKGROUND OF THE INVENTION

The interferons are a family of glycoproteins whose secretion from cells is induced by a number of signals including viruses, double-stranded RNAs, other polynucleotides, antigens, and mitogens. Interferons exhibit multiple biological activities, including antiviral, antiproliferative, and immunomodulatory activities. At least three distinct types of human interferons, α, β, and γ, have been distinguished based on a number of factors, including anti-viral and anti-proliferative activities.

α-interferons act through interaction with cell-surface receptors and induce the expression, primarily at the transcriptional level, of a broad but specific set of cellular genes. Several INTERFERON-induced gene products have been used as markers for the biological activity of interferons. These include, for instance, ISG15, ISG54, IRF1, GBP, and IP10.

Assays for interferon-mediated anti-viral activity have been described in the art. See, for example, McNeill, (1981) *J Immunol Methods*. 46:121-7. Assays for interferon antiviral activity include inhibition of cytopathic effect, virus plaque formation; and reduction of virus yield. Viral cytopathic effect assays measure the degree of protection induced in cell cultures pretreated with interferon INTERFERON and subsequently infected with viruses. See, for example, Rubinstein *et al.* (1981) *J Virol.* 37:755-8. Plaque-reduction assays can be used to measure the resistance of INTERFERON-treated cell cultures to a plaque-forming

virus (for instance, measles virus). Finally, virus yield assays measure the amount of virus released from cells during, for instance, a single growth cycle. Such assays are useful for testing the antiviral activity of INTERFERONs against viruses that do not cause cytopathic effects, or that do not build plaques in target-cell cultures.

α-2b interferons have since been shown to be efficacious against viral, proliferative, and inflammatory disorders, including malignant melanoma, hairy cell leukemia, chronic hepatitis B, chronic hepatitis C, condylomata acuminata, follicular (non-Hodgkin's) lymphoma, and AIDS-related Kaposi's sarcoma. Clinical uses of interferons are reviewed in Gresser (1997) *J. Leukoc. Biol.* 61:567-74, and Pfeffer (1997) *Semin Oncol.* 24(3 Suppl 9):S9-63-S9-69.

SUMMARY OF THE INVENTION

The present invention provides biologically active variants of human α-2b-interferon. The variants contain carboxy terminus truncations when compared with the amino acid sequence of full-length human α-2b-interferon. It is the novel finding of the present invention that these truncated variants have the biological activity of human α-2b-interferon. The sequences of the α-2b-interferon variant precursor polypeptides are shown in SEQ ID NOS:1-5, while the sequences of the mature α-2b-interferon variant polypeptides s are shown in SEQ ID NOS:6-10. Accordingly, in one embodiment, the invention provides a purified polypeptide consisting of an amino acid sequence selected from the amino acid sequences shown in SEQ ID NOS:1-10.

In some embodiments, the polypeptide consisting of a signal peptide operably linked to an amino acid sequence selected from the sequences shown in SEQ ID NOS:6-10. In some embodiments, the signal peptide is a mammalian signal peptide, while in other embodiments, the signal peptide is a plant signal peptide.

In one aspect of the invention, the polypeptides of the invention are recombinantly produced in a host cell plant cell. In particular embodiments, the host cell is a mammalian cell, a plant cell, a yeast cell, an insect cell, or a prokaryotic cell.

The invention also encompasses polynucleotides encoding the polypeptide of the invention, expression cassettes comprising these polynucleotides, and host cells comprising the expression cassettes.

In another embodiment, the invention provides a composition comprising a purified polypeptide of the invention and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1

Figure 1 show the interferon levels (as determined by a solid phase sandwich immunoassay) in the media and tissue of a transformed duckweed culture, as described Example 1.

Figure 2

Figure 2 show the interferon levels (as determined by a solid phase sandwich immunoassay) in the media and tissue of a transformed duckweed cultures, as described in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides biologically active variants of human α-2b-interferon. The variants contain carboxy terminus truncations when compared with the amino acid sequence of full-length human α-2b-interferon. It is the novel finding of the present invention that these truncated variants have the biological activity of human α-2b-interferon. The present invention provides the sequences of these α-2b interferon variants.

Definitions:

An "isolated" or "purified" polynucleotide or polypeptide is substantially or essentially free from components that normally accompany or interact with the polynucleotide or protein as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide or polypeptide is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or

substantially free of chemical precursors or other chemicals when chemically synthesized. Optimally, an "isolated" polynucleotide is free of sequences (optimally protein encoding sequences) that naturally flank the polynucleotide (i.e., sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, optimally culture medium represents less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-polypeptide-of-interest chemicals.

A "biologically active polypeptide" refers to a polypeptide that has the capability of performing one or more biological functions or a set of activities normally attributed to the polypeptide in a biological context. Those skilled in the art will appreciate that the term "biologically active" includes polypeptides in which the biological activity is altered as compared with the native protein (e.g., suppressed or enhanced), as long as the protein has sufficient activity to be of interest for use in industrial or chemical processes or as a therapeutic, vaccine, or diagnostics reagent. Biological activity can be determined by any method available in the art. For example, the biological activity of members of the interferon family of proteins can be determined by any of a number of methods including their interaction with interferon-specific antibodies, their ability to increase resistance to viral infection, or their ability to modulate the transcription of interferon-regulated gene targets. Examples of such methods are described elsewhere herein.

The terms "expression" or "production" refer to the biosynthesis of a gene product, including the transcription, translation, and assembly of said gene product.

By "recombinantly produced" is intended a polypeptide that has been prepared by recombinant DNA techniques. Recombinantly produced interferon variants can be produced by culturing a host cell transformed with an expression cassette comprising a polynucleotide that encodes an α-interferon variant of the invention. The host cell is one that can transcribe the nucleotide sequence and

produce the desired protein, and can be prokaryotic (for example, *E. coli*) or eukaryotic (for example a plant, yeast, insect, or mammalian cell).

The term "duckweed" refers to members of the family Lemnaceae. This family currently is divided into five genera and 38 species of duckweed as follows: genus *Lemna* (*L. aequinoctialis*, *L. disperma*, *L. ecuadoriensis*, *L. gibba*, *L. japonica*, *L. minor*, *L. miniscula*, *L. obscura*, *L. perpusilla*, *L. tenera*, *L. trisulca*, *L. turionifera*, *L. valdiviana*); genus *Spirodela* (*S. intermedia*, *S. polyrrhiza*); genus *Wolffia* (*Wa. angusta*, *Wa. arrhiza*, *Wa. australina*, *Wa. borealis*, *Wa. brasiliensis*, *Wa. columbiana*, *Wa. elongata*, *Wa. globosa*, *Wa. microscopica*, *Wa. neglecta*) genus *Wolfiella* (*Wl. caudata*, *Wl. denticulata*, *Wl. gladiata*, *Wl. hyalina*, *Wl. lingulata*, *Wl. repunda*, *Wl. rotunda*, and *Wl. neotropica*), and genus *Landoltia* (*L. punctata)*. Any other genera or species of Lemnaceae, if they exist, are also aspects of the present invention. *Lemna* species can be classified using the taxonomic scheme described by Les *et al.* (2002) *Systematic Botany* 27:221-40.

"Operably linked" as used herein in reference to nucleotide sequences refers to multiple nucleotide sequences that are placed in a functional relationship with each other. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame.

A. Polypeptides.

The present invention identifies biologically active variants of human α-2b interferon. These variants contain carboxy terminus truncations of 4-8 amino acids in comparison with full-length human α-2b-interferon. The sequences of the mature forms of these biologically active interferon variants are provided in SEQ ID NOS:6-10, while the corresponding sequences of the interferon variant precursor polypeptides are provided in SEQ ID NOS:1-5.

In some embodiments, the invention encompasses compositions comprising a mixture of two or more α-interferon variants of the invention. Such mixtures may comprise two or more, three or more, four or more, five or more, or more than six of the α-interferon variants consisting of the amino acid sequences set forth in SEQ ID NOS:1-10.

B. Polynucleotides and Expression Cassettes

In one aspect the present invention provides polynucleotides encoding the biologically active α-interferons of the invention. Accordingly, the invention encompasses polynucleotides encoding polypeptides consisting of the amino acid sequences set forth in SEQ ID NOS:1-10.

In some embodiments, the polynucleotides may be comprised within an expression cassette. The expression cassette comprises a transcriptional initiation region linked to the nucleic acid or gene of interest. Such an expression cassette can be provided with a plurality of restriction sites for insertion of the polynucleotide of interest to be under the transcriptional regulation of the regulatory regions.

The transcriptional initiation region, (e.g., a promoter) may be native or homologous or foreign or heterologous to the host, or could be the natural sequence or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

Any suitable promoter known in the art can be employed according to the present invention (including bacterial, yeast, fungal, insect, mammalian, and plant promoters). For example, plant promoters may be used. Exemplary promoters include, but are not limited to, the Cauliflower Mosaic Virus 35S promoter, the opine synthetase promoters (e.g., nos, mas, ocs, etc.), the ubiquitin promoter, the actin promoter, the ribulose bisphosphate (RubP) carboxylase small subunit promoter, and the alcohol dehydrogenase promoter. The duckweed RubP carboxylase small subunit promoter is known in the art (Silverthorne *et al.* (1990) *Plant Mol. Biol.* 15:49). Other promoters from viruses that infect plants, preferably duckweed, are also suitable including, but not limited to, promoters isolated from Dasheen mosaic virus, Chlorella virus (e.g., the Chlorella virus adenine methyltransferase promoter; Mitra *et al.* (1994) *Plant Mol. Biol.* 26:85), tomato spotted wilt virus, tobacco rattle virus, tobacco necrosis virus, tobacco ring spot virus, tomato ring spot virus, cucumber mosaic virus, peanut stump virus, alfalfa mosaic virus, sugarcane baciliform badnavirus and the like.

The overall strength of a given promoter can be influenced by the combination and spatial organization of cis-acting nucleotide sequences such as upstream activating sequences. For example, activating nucleotide sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene can enhance transcription from the *Agrobacterium tumefaciens* mannopine synthase promoter (see U.S. Patent 5,955,646 to Gelvin *et al.*). In the present invention, the expression cassette can contain activating nucleotide sequences inserted upstream of the promoter sequence to enhance the expression of the nucleotide sequence of interest. In one embodiment, the expression cassette includes three upstream activating sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene operably linked to a promoter derived from an Agrobacterium tumefaciens mannopine synthase gene (see U.S Patent 5,955,646, herein incorporated by reference).

The expression cassette may include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a polynucleotide of interest, and a transcriptional and translational termination region functional in plants. Any suitable termination sequence known in the art may be used in accordance with the present invention. The termination region may be native with the transcriptional initiation region, may be native with the nucleotide sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid *of A. tumefaciens*, such as the octopine synthetase and nopaline synthetase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141; Proudfoot (1991) *Cell* 64:671; Sanfacon *et al.* (1991) *Genes Dev.* 5:141; Mogen *et al.* (1990) *Plant Cell* 2:1261; Munroe *et al.* (1990) *Gene* 91:151; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627. Additional exemplary termination sequences are the pea RubP carboxylase small subunit termination sequence and the Cauliflower Mosaic Virus 35S termination sequence. Other suitable termination sequences will be apparent to those skilled in the art.

Alternatively, the polynucleotides of interest can be provided on any other suitable expression cassette known in the art.

The expression cassettes may contain more than one polynucleotide to be transferred and expressed in the transformed plant. Thus, each nucleic acid

sequence will be operably linked to 5' and 3' regulatory sequences. Alternatively, multiple expression cassettes may be provided.

The expression cassette may comprise a selectable marker gene for the selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. See DeBlock *et al.* (1987) *EMBO J.* 6:2513; DeBlock *et al.*(1989) *Plant Physiol.* 91:691; Fromm *et al.* (1990) *BioTechnology* 8:833; Gordon-Kamm *et al.* (1990) *Plant Cell* 2:603; and Frisch *et al.* (1995) *Plant Mol. Biol.* 27:405-9. For example, resistance to glyphosphate or sulfonylurea herbicides has been obtained using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, boromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides.

For purposes of the present invention, selectable marker genes include, but are not limited to, genes encoding neomycin phosphotransferase II (Fraley *et al.* (1986) *CRC Critical Reviews in Plant Science* 4:1); neomycin phosphotransferase III (Frisch *et al.* (1995) *Plant Mol. Biol.* 27:405-9); cyanamide hydratase (Maier-Greiner *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:4250); aspartate kinase; dihydrodipicolinate synthase (Perl *et al.* (1993) *BioTechnology* 11:715); bar gene (Toki *et al.* (1992) *Plant Physiol.* 100:1503; Meagher *et al.* (1996) *Crop Sci.* 36:1367); tryptophan decarboxylase (Goddijn *et al.* (1993) *Plant Mol. Biol.* 22:907); neomycin phosphotransferase (NEO; Southern *et al.* (1982) *J. Mol. Appl. Gen.* 1:327); hygromycin phosphotransferase (HPT or HYG; Shimizu *et al.* (1986) *Mol. Cell. Biol.* 6:1074); dihydrofolate reductase (DHFR; Kwok *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:4552); phosphinothricin acetyltransferase (DeBlock *et al.* (1987) *EMBO J.* 6:2513); 2,2-dichloropropionic acid dehalogenase (Buchanan-Wollatron *et al.* (1989) *J. Cell. Biochem.* 13D:330); acetohydroxyacid synthase

(U.S. Pat. No. 4,761,373 to Anderson *et al.*; Haughn *et al.* (1988) *Mol. Gen. Genet.* 221:266); 5-enolpyruvyl-shikimate-phosphate synthase (aroA; Comai *et al.* (1985) *Nature* 317:741); haloarylnitrilase (WO 87/04181 to Stalker *et al.*); acetyl-coenzyme A carboxylase (Parker *et al.* (1990) *Plant Physiol.* 92:1220); dihydropteroate synthase (sulI; Guerineau *et al.* (1990) *Plant Mol. Biol.* 15:127); and 32 kDa photosystem II polypeptide (psbA; Hirschberg *et al.* (1983) *Science* 222:1346 (1983).

Also included are genes encoding resistance to: gentamycin (*e.g.*, aacC1, Wohlleben *et al.* (1989) *Mol. Gen. Genet.* 217:202-208); chloramphenicol (Herrera-Estrella *et al.* (1983) *EMBO J.* 2:987); methotrexate (Herrera-Estrella *et al.* (1983) *Nature* 303:209; Meijer *et al.* (1991) *Plant Mol. Biol.* 16:807); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol.* 5:103; Zhijian *et al.* (1995) *Plant Science* 108:219; Meijer *et al.* (1991) *Plant Mol. Bio.* 16:807); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet.* 210:86); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res.* 5:131); bleomycin (Hille *et al.* (1986) *Plant Mol. Biol.* 7:171); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Bio.* 15:127); bromoxynil (Stalker *et al.* (1988) *Science* 242:419); 2,4-D (Streber *et al.* (1989) *BioTechnology* 7:811); phosphinothricin (DeBlock *et al.* (1987) EMBO J. 6:2513); spectinomycin (Bretagne-Sagnard and Chupeau, *Transgenic Research* 5:131).

The bar gene confers herbicide resistance to glufosinate-type herbicides, such as phosphinothricin (PPT) or bialaphos, and the like. As noted above, other selectable markers that could be used in the vector constructs include, but are not limited to, the pat gene, also for bialaphos and phosphinothricin resistance, the ALS gene for imidazolinone resistance, the HPH or HYG gene for hygromycin resistance, the EPSP synthase gene for glyphosate resistance, the Hm1 gene for resistance to the Hc-toxin, and other selective agents used routinely and known to one of ordinary skill in the art. See Yarranton (1992) *Curr. Opin. Biotech.* 3:506; Chistopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314; Yao *et al.* (1992) *Cell* 71:63; Reznikoff (1992) *Mol. Microbiol.* 6:2419; Barkley *et al.* (1980) *The Operon* 177-220; Hu *et al.* (1987) *Cell* 48:555; Brown *et al.* (1987) *Cell* 49:603; Figge *et al.* (1988) *Cell* 52:713; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549; Deuschle *et al.* (1990) *Science* 248:480; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343; Zambretti *et*

al. (1992) *Proc. Natl. Acad. Sci. USA* 89:3952; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072; Wyborski *et al.* (1991) *Nuc. Acids Res.* 19:4647; Hillenand-Wissman (1989) *Topics in Mol. And Struc. Biol.* 10:143; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094; Gatz *et al.* (1992) *Plant J.* 2:397; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology* 78; and Gill *et al.* (1988) *Nature* 334:721. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes are not meant to be limiting. Any lethal or non-lethal selectable marker gene can be used in the present invention.

C. Modification of Nucleotide Sequences for Enhanced Expression in a Host Cell

The present invention provides for the modification of the polynucleotide to enhance its recombinant production in a host cell. One such modification is the synthesis of the nucleotide sequence of interest using codons preferred in the host cell. Methods are available in the art for synthesizing nucleotide sequences with host-preferred codons. See, e.g., U.S. Patent Nos. 5,380,831 and 5,436,391; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 15:3324; Iannacome *et al.* (1997) *Plant Mol. Biol.* 34:485; and Murray *et al.*, (1989) *Nucleic Acids. Res.* 17:477, herein incorporated by reference. The preferred codons may be determined from the codons of highest frequency in the proteins expressed in the host cell. All or any part of the polynucleotide may be optimized or synthetic. In other words, fully optimized or partially optimized sequences may also be used. For example, 40 %, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the codons may be host cell-preferred codons. In one embodiment, between 90 and 96 % of the codons are host cell-preferred codons.

Other modifications can also be made to the nucleotide sequence of interest to enhance its expression in a host cell. These modifications include, but are not limited to, elimination of sequences encoding spurious polyadenylation signals,

exon-intron splice site signals, transposon-like repeats, and other such well characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence may be modified to avoid predicted hairpin secondary mRNA structures.

Expression of a transgene in a host cell can also be enhanced by the use of 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include, but are not limited to, picornavirus leaders, e.g., EMCV leader (Encephalomyocarditis 5' noncoding region; Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci USA* 86:6126); potyvirus leaders, e.g., TEV leader (Tobacco Etch Virus; Allison *et al.* (1986) *Virology* 154:9); human immunoglobulin heavy-chain binding protein (BiP; Macajak and Samow (1991) *Nature* 353:90); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4; Jobling and Gehrke (1987) *Nature* 325:622); tobacco mosaic virus leader (TMV; Gallie (1989) *Molecular Biology of RNA*, 23:56); potato etch virus leader (Tomashevskaya *et al.* (1993) *J. Gen. Virol.* 74:2717-2724); Fed-1 5' untranslated region (Dickey (1992) *EMBO J.* 11:2311-2317); RbcS 5' untranslated region (Silverthorne *et al.* (1990) *J. Plant. Mol. Biol.* 15:49-58); and maize chlorotic mottle virus leader (MCMV; Lommel *et al.* (1991) Virology 81:382). See also, Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965. Leader sequence comprising plant intron sequence, including intron sequence from the maize dehydrogenase 1 gene, the castor bean catalase gene, or the Arabidopsis tryptophan pathway gene PAT1 has also been shown to increase translational efficient in plants (Callis *et al.* (1987) *Genes Dev.* 1:1183-1200; Mascarenhas *et al.* (1990) *Plant Mol. Biol.* 15:913-920). Other leader sequences that may be used include the leader from the *Lemna gibba* ribulose-bis-phosphate carboxylase small subunit 5B gene (Buzby *et al.* (1990) *Plant Cell* 2:805-814).

D. Signal Peptides

Secreted proteins including interferon are usually translated from precursor polypeptides that include a "signal peptide" that interacts with a receptor protein on the membrane of the endoplasmic reticulum to direct the translocation of the

growing polypeptide chain across the membrane and into the endoplasmic reticulum for secretion from the cell. This signal peptide is often cleaved from the precursor polypeptide to produce a "mature" polypeptide lacking the signal peptide. In an embodiment of the present invention, a biologically active interferon variant is expressed in duckweed from a polynucleotide that is operably linked with a nucleotide sequence encoding a signal peptide that directs secretion of the interferon variant from the host cell. Any signal peptide known in the art can be used according to the present invention. Plant signal peptides that target protein translocation to the endoplasmic reticulum (for secretion into the apoplast or outside of the cell) are known in the art. See, for example, U.S. Patent No. 6,020,169 to Lee *et al.* Alternatively, a mammalian signal peptide can be used to target recombinant interferon variants expressed in a host cell for secretion. In one embodiment of the present invention, the mammalian signal peptide that targets polypeptide secretion is the human α-2b-interferon signal peptide (amino acids 1-23 of NCBI Protein Accession No. AAB59402 and SEQ ID NO:12).

In one embodiment, the nucleotide sequence encoding the signal peptide is modified for enhanced expression in the host cell, utilizing any modification or combination of modifications disclosed in section C above for the polynucleotides of interest.

The secreted biologically active polypeptide can be harvested from the host cell or host cell culture medium by any conventional means known in the art and purified by chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like.

E. Host cells

In some embodiments, the invention encompasses host cells containing the expression cassettes of the invention. These host cells may be used to recombinantly produce the α-interferon variants. The host cell is one that can transcribe the polynucleotide and can be prokaryotic (for example, *E. coli*) or eukaryotic (for example a plant, yeast, insect, or mammalian cell). Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in*

Enzymology 185, Academic Press, San Diego, CA (1990). Methods of transforming such host cells with a nucleic acid molecule are well known in the art.

In some embodiments, the host cells are plant cells. Both monocot cells and dicot cells may be used. Suitable methods of introducing polynucleotides into plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058).

In particular embodiments, the host cells for recombinant expression of the α-interferon variants are duckweed cells. Stably transformed duckweed cells may also be obtained according any method known in the art. See, for example, U.S. patent No. 6,040,498, and PCT publications WO0210414 and WO02097433.

When the host cell is a plant cell, transgenic plants can be regenerated from transformed host cells.

F. Pharmaceutical Compositions

The α-interferon variants of the invention can be incorporated into pharmaceutical compositions. Such compositions typically include one or more α-interferon variant polypeptides and a pharmaceutically acceptable carrier. The phrase "pharmaceutically acceptable carrier" as used herein is a carrier that is conventionally used in the art to facilitate the storage, administration, and/or the healing effect of the therapeutic ingredients. A carrier may also reduce any undesirable side effects of the α-interferon.

A carrier should be stable (*i.e.*, incapable of reacting with other ingredients in the composition), and it should not produce adverse effects in patients at the dosages and concentrations employed for treatment. Suitable carriers include large stable macromolecules such as albumin, gelatin, collagen, polysaccharide, monosaccharides, polyvinyl-pyrrolidone, polylactic acid, polyglycolic acid, polymeric amino acids, fixed oils, ethyl oleate, liposomes, glucose, sucrose, lactose, mannose, dextrose, dextran, cellulose, sorbitol, polyethylene glycol (PEG), and the like. Slow-release carriers, such as hyaluronic acid, may also be suitable.

Other acceptable components in the composition include, but are not limited to, pharmaceutically acceptable agents that modify isotonicity including water, salts, sugars, polyols, amino acids, and buffers. Examples of suitable buffers include phosphate, citrate, succinate, acetate, and other organic acids or their salts and salts that modify the tonicity such as sodium chloride, sodium phosphate, sodium sulfate, potassium chloride, and can also include the buffers listed above.

The pharmaceutical composition may additionally comprise a solubilizing agent or solubility enhancer. Examples of such solubility enhancers are described, for example, in Wang *et al.* (1980) *J. Parenteral Drug Assoc.* 34:452-462; herein incorporated by reference.

Non-limiting examples of solubilizing agents encompassed by the present invention include surfactants (detergents) that have a suitable hydrophobic-hydrophilic balance to solubilize interferon. Strong natural or synthetic anionic surfactants such as alkali metal salts of fatty acids and alkali metal alkyl sulfates may be used. Examples of other solubilizing agents that can be used in compositions of the invention include but are not limited to sodium dodecyl sulfonate, sodium decyl sulfate, sodium tetradecyl sulfate, sodium tridecyl

sulfonate, sodium myristate, sodium caproylate, sodium dodecyl N-sarcosinate, and sodium tetradecyl N-sarcosinate. Classic stabilization of pharmaceuticals by surfactants or emulsifiers is described, for example, in Levine *et al.* (1991) *J. Parenteral Sci. Technol.* 45(3):160-165. Additional suitable surfactants are discussed in U.S. Patent No. 5,935,566, herein incorporated by reference.

In addition to those agents disclosed above, other stabilizing agents, such as ethylenediaminetetracetic acid (EDTA) or one of its salts such as disodium EDTA, can be added to further enhance the stability of the pharmaceutical compositions. The EDTA acts as a scavenger of metal ions known to catalyze many oxidation reactions, thus providing an additional stabilizing agent.

Where the composition is used for delivery to a mammal such as a human, the isotonicity of the composition is also a consideration. Thus, in one embodiment, the composition for an injectable solution will provide an isotonicity the same as, or similar to, that of patient serum or body fluids. To achieve isotonicity, a salt, such as sodium chloride, potassium, chloride, or a phosphate buffer, can be added to the solution at an appropriate concentration.

The pH of the composition is also a consideration. The compositions of the invention have a pH ranging from about 4.0 to about 8.5. Suitable pH ranges include, for example, about 4.5 to about 7.8 or about 5.0 to about 7.5 such as about 6.0, about 6.2, about 6.4, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, or about 7.6.

A thorough discussion of formulation and selection of pharmaceutically acceptable carriers, stabilizers, etc. can be found in *Remington's Pharmaceutical Sciences* (1990) (18th ed., Mack Pub. Co., Eaton, Pennsylvania), herein incorporated by reference.

EXPERIMENTAL

The following examples are offered for purposes of illustration, not by way of limitation.

Expression Vectors

The expression vector pBMSP-1 used in some of the examples is described in U.S. Patent No. 5,955,646, herein incorporated by reference. The pBMSP-1 transcriptional cassette contains three copies of a transcriptional activating nucleotide sequence derived from the *Agrobacterium tumefaciens* octopine synthase and, an additional transcriptional activating nucleotide sequence derived from the *Agrobacterium tumefaciens* mannopine synthase gene, a promoter region derived from the *Agrobacterium tumefaciens* mannopine synthase gene, a polylinker site for insertion of the nucleotide sequence encoding the polypeptide of interest, and a termination sequence derived from the *Agrobacterium tumefaciens* nopaline synthase gene (see, van Engelen *et al.* (1995) 4:288-290; Ni *et al.* (1995) *Plant J.* 7:661-76; and Luehrsen *et al.* (1991) *Mol. Gen. Genet.* 225:81-93, each of which is herein incorporated by reference). The pBMSP-1 expression vector also contains a nucleotide sequence coding for neomycin phosphotransferase II as a selectable marker. Transcription of the selectable marker sequence is driven by a promoter derived from the *Agrobacterium tumefaciens* nopaline synthase gene.

The expression vector pBMSP-3, also used in some of the following examples, contains the components of the pBMSP-1 expression vector described above and additionally contains a nucleotide sequence corresponding to nucleotides 1222-1775 of the maize alcohol dehydrogenase gene (GenBank Accession Number X04049) inserted between the promoter and the polylinker.

Expression Constructs for the Production of Human α-2b-Interferon in Duckweed

Table 2 shows the expression constructs used for the production of human α-interferon in duckweed.

Table 2

Construct Name	Expression Vector	Signal Peptide	Interferon-encoding Sequence
IFN01	pBMSP-1	None	Non-optimized human α-2b-interferon
IFN02	pBMSP-3	Non-optimized human α-2b-interferon signal peptide	Non-optimized human α-2b-interferon
IFN03	pBMSP-3	*Arabidopsis thaliana* endochitinase signal peptide (nucleotides 338-399 of GenBank Accession number AB023460 with an additional "A" added to the 3' end of the sequence)	Non-optimized human α-2b-interferon
IFN05	pBMSP-3	Modified rice α-amylase signal peptide*	Non-optimized human α-2b-interferon
IFN07	pBMSP-3	Wild type rice α-amylase signal peptide (nucleotides 34-126 of GenBank Accession No. M24286)	Non-optimized human α-2b-interferon
IFN08	pBMSP-3	Duckweed codon optimized wild type rice α-amylase signal peptide	Non-optimized human α-2b-interferon
IFN09	pBMSP-3	Duckweed codon optimized wild type rice α-amylase signal peptide	Duckweed codon optimized human α-2b-interferon
IFN10	pBMSP-3	None	Duckweed codon optimized human α-2b-interferon
IFN11	pBMSP-1	Duckweed codon optimized wild type rice α-amylase signal peptide	Duckweed codon optimized human α-2b-interferon
IFN12	pBMSP-1	None	Duckweed codon optimized human α-2b-interferon
IFN053	modified pBMSP-3**	Duckweed codon optimized wild type rice α-amylase signal peptide	Duckweed codon optimized human α-2b-interferon

*The nucleotide sequence encoding the modified rice α-amylase signal peptide corresponds to nucleotides 34-126 of NCBI Accession No. M24286, except that nucleotides 97-102 have been changed from "CTTGGC" to "ATCGTC."

**For construct IFN053, the 5'-mas leader in pBMPSP3 was replaced with the leader from the ribulose-bis-phosphate carboxylase small subunit 5B gene of *Lemna gibba* (nucleotides 689-751 of NCBI Accession No. S45167, Buzby *et al.* (1990) *Plant Cell* 2:805-814).

Transformation of Duckweed

Duckweed fronds or duckweed nodule cultures (derived from *Lemna minor* strain 8627 in these examples) were transformed with the expression constructs described above using Agrobacteria-mediated transformation methods. *Agrobacterium tumefaciens* strain C58Z707, a disarmed, broad host range C58 strain (Hepburn *et al.* (1985) *J. Gen. Microbiol. 131*:2961-2969) is used for transformation in these examples. The expression constructs described above were mobilized into *A. tumefaciens* by electroporation, or by a triparental mating procedure using *E. coli* MM294 harboring the mobilizing plasmid pRK2013 (Hoekema *et al.* (1983) *Nature 303*: 179-180; Ditta *et al.* (1980) *Proc. Natl. Acad. Sci. USA 77*: 7347-7350). C58Z707 strains comprising the expression constructs described above are streaked on AB minimal medium (Chilton *et al.*, (1974) *Proc. Nat. Acad. Sci. USA 71*: 3672 -3676) or in YEB medium (1 g/L yeast extract, 5 g/L beef extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgSO_4$) containing streptomycin at 500 mg/L, spectinomycin at 50 mg/L and kanamycin sulfate at 50 mg/L and grown overnight at 28° C.

In these examples, *Lemna minor* strain 8627 was used for transformation although any *Lemna* strain can be used. Fronds were grown on liquid Schenk and Hildebrandt medium (Schenk and Hildebrandt (1972) *Can. J. Bot. 50*:199) containing 1% sucrose and 10 μM indoleacetic acid at 23° C. under a 16-hour light/8-hour dark photoperiod with light intensity of approximately 40 $\mu M/m^2 \cdot sec$. For inoculation, individual fronds were separated from clumps and floated in inoculation media for approximately 2 to 20 minutes. The inoculating medium is Schenk and Hildebrandt medium (pH 5.6) supplemented with 0.6 M mannitol and 100 μM acetosyringone, with the appropriate *Agrobacterium tumefaciens* strain comprising the expression construct present at a concentration of about 1 X 10^9cells/ml. These fronds were then transferred to Schenk and Hildebrandt medium (pH 5.6) containing 1% sucrose, 0.9% agar, and 20 mg/L acetosyringone and are co-cultivated for 3 or 4 days in the dark at 23° C.

Following co-cultivation, the fronds were transferred for recovery to Schenk and Hildebrandt medium or Murashige and Skoog medium (Murashige and Skoog (1962) *Physiol. Plant. 15*:473) supplemented with 200 μg/ml kanamycin

sulfate. Fronds wre decontaminated from infecting Agrobacteria by transferring the infected tissue to fresh medium with antibiotic every 2-4 days. The fronds were incubated on this medium for approximately four weeks under conditions of low light (1-5 $\mu M/m^2 \cdot sec$).

Duckweed nodule cultures for transformation were produced as follows. Duckweed fronds are separated, the roots are cut off with a sterile scalpel, and the fronds are placed, ventral side down, on Murashige and Skoog medium (catalog number M-5519; Sigma Chemical Corporation, St. Louis, MO) pH 5.6, supplemented with 5 μM 2,4-dichlorophenoxyacetic acid, 0.5 μM 1-Phenyl-3(1,2,3-thiadiazol-5-yl) urea thidiazuron (Sigma P6186), 3% sucrose, 0.4 Difco Bacto-agar (Fisher Scientific), and 0.15% Gelrite (Sigma). Fronds are grown for 5-6 weeks. At this time, the nodules (small, yellowish cell masses) appeared, generally from the central part of the ventral side. This nodule tissue was detached from the mother frond and cultured in Murashige and Skoog medium supplemented with 3% sucrose, 0.4% Difco Bacto-agar, 0.15% Gelrite, 1 μM 2,4-dichlorophenoxyacetic acid, and 2 μM benzyladenine.

Duckweed nodule cultures were transformed as follows. The appropriate *Agrobacterium tumefaciens* strain is grown on potato dextrose agar or YEB agar with 50 mg/L kanamycin and 100 μM acetosyringone, and resuspended in Murashige and Skoog medium supplemented with 0.6 M Mannitol and 100 μM acetosyringone. Nodule culture tissue was inoculated by immersing in the solution of resuspended bacteria for 1-2 minutes, blotted to remove excess fluid, and plated on co-cultivation medium consisting of Murashige and Skoog medium supplemented with auxin and cytokinin optimized to promote nodule growth and 100 μM acetosyringone. *See*, Yamamoto *et al.* (2001) *In Vitro Cell Dev. Biol. Plant* 37:349-353.

For selection, nodule culture tissue was transferred to regeneration medium Murashige and Skoog medium with 3% sucrose, 1 μM 2,4-dichlorophenoxyacetate, 2 μM benzyladenine, 0.4 % Difco Bacto-Agar, 0.15% Gelrite 500 mg/L cefotaxime, and 200 mg/L kanamycin sulfate and cultured for approximately four weeks under continuous light (20-40 $\mu M/m^2 \cdot sec$). The nodule tissue was transferred every 7 days to fresh culture medium. Selection is complete

when the nodule tissue shows vigorous growth on the selection agent. In some examples, the transformed duckweed nodule cultures are transferred directly to regeneration medium for selection, instead of undergoing selection in co-cultivation medium.

For regeneration of transformed duckweed, the selected nodule culture was transferred to regeneration medium (0.5 X Schenk and Hildebrandt medium supplemented with 1% sucrose and 200 mgs/L kanamycin) to organize and produce plants. The nodule culture is incubated on regeneration medium under full light for approximately 3 weeks, until fronds appear. Fully organized fronds were transferred to liquid Schenk and Hildebrandt medium with 1-3% sucrose and incubated under full light for further clonal proliferation.

Detection of Biologically-Active Interferon Produced from Duckweed Fronds or Duckweed Nodule Culture

Biologically-active interferon was detected by various assays, including a solid phase sandwich immunoassay as described in Staehlin *et al.* (1981) *Methods Enzymol. 79*:589-594 and Kelder *et al.* (1986) *Methods Enzymol. 119*:582-587, herein incorporated by reference, and a cytopathic effect inhibition assay (described in Tovey *et al.* (1978) *Nature 276*:270-272, herein incorporated by reference. Secreted interferon was collected from the duckweed culture medium, while non-secreted interferon was collected from ground-up or lysed duckweed plants or duckweed nodule tissue.

A solid phase sandwich immunoassay for interferon was performed using a kit from PBL Laboratories (New Brunswick, NJ) according to the manufacturer's instructions. Briefly, interferon is captured by an antibody bound to the microtiter plate wells. A second antibody is then used to reveal the bound antibody. An anti-secondary antibody conjugated to horseradish peroxidase (HRP) is then used to mark the interferon. Tetramethyl benzidine (TMB) initiates a peroxidase-catalyzed color change so that the interferon level can be observed and compared with a standard. A monoclonal antibody specific for α-2b-interferon (Cat. No. 11105, PBL Laboratories) was used for this assay in the present examples.

A cytopathic effect inhibition assay was performed according to the methods of Tovey *et al.* (1978) *Nature 276*:270-272. Briefly, serial two-fold

dilutions of the preparation to be assayed are diluted in a 96 well microtiter plate (Falcon Inc) in a volume of 100 µl of Eagles minimal essential medium (Life Technologies Inc) supplemented with 2 % fetal calf serum (Life Technologies Inc) in parallel with serial two fold dilutions of the US National Institutes of Health human IFN alpha international reference preparation (G-002-901-527). Twenty thousand human amnion cells (line WISH) are then added to each well of the microtiter plate in a volume of 100 µl of medium with 2 % fetal calf serum. The cells were incubated over-night in an atmosphere of 5 % CO_2 in air at 37°C, the medium was removed and replaced with 200 µl of medium with 2 % fetal calf serum containing vesicular stomatitis virus at a multiplicity of infection of 0.1. The cells were further incubated over-night in an atmosphere of 5 % CO_2 in air at 37 °C and the cytopathic effect due to virus replication was then evaluated under a light microscope. Interferon titers were expressed as the reciprocal of the IFN dilution which gave 50 % protection against the cytopathic effects of the virus. Interferon titers were expressed in international reference units by reference to the titer of the reference preparation.

The following examples demonstrate the expression of biologically active interferon variants in duckweed.

Example 1

A study was performed to determine culture IFN levels in media and tissue at various time points in a batch culture. A set of 20-30ml 175oz.-culture jars were inoculated on Day 0 with 20 fronds of a line previously identified as expressing detectable levels of human α-2b-interferon (IFN). The cultures were grown under autotrophic, buffered conditions with continuous high light provided by plant/aquarium fluorescent grow bulbs. At each time point - days 5, 7, 13, 15, and 18 – the fresh weight and media volume were measured for four cultures. From each culture, media and tissue samples were obtained and a plant protease inhibitor cocktail was added. The tissue samples were ground and spun cold. The supernatant was collected. Media and tissue extracts were stored at –70°C until all samples were collected. IFN levels in media and tissue extracts were determined on the same day using the solid phase sandwich immunoassay described above.

Total culture IFN in media and tissue was calculated by multiplying measured IFN concentrations and the volume of media and the fresh weight for the culture, respectively. Figure 1 shows the relative IFN levels on days 7, 13, 15, and 18 compared to day 5. The last time point represents the average value for three cultures instead of four due to loss of one culture.

Example 2

A study was performed to determine culture IFN levels in media and tissue at various time points in a batch culture. A set of 24-30ml 175oz.-culture jars were inoculated on Day 0 with 20 fronds of the same line as in Example 1. The cultures were grown under autotrophic, unbuffered conditions with continuous high light provided by plant/aquarium fluorescent grow bulbs. At each time point - days 7, 10, 12, 14, 17, and 19 – the fresh weight and media volume were measured for four cultures. From each culture, media and tissue samples were obtained and a plant protease inhibitor cocktail was added. The tissue samples were ground and spun cold. The supernatant was collected. Media and tissue extracts were stored at –70°C until all samples were collected. IFN levels in media and tissue extracts were determined on the same day using the solid phase sandwich immunoassay described above. Total culture IFN in media and tissue was calculated by multiplying measured IFN concentrations and the volume of media and the fresh weight for the culture, respectively. Figure 2 shows the relative IFN levels on days 14, 17, and 19 compared to day 12. Media IFN levels on day 7 and day 10 were below the range of the extended range protocol for the immunoassay.

Example 3

Duckweed lines transformed with the expression constructs listed in Table 2 were produced using the methods described above. These transformed duckweed lines were grown for 14 days under autotrophic conditions. Bovine serum albumin at a concentration of 0.2 mg/ml was included in the growth media. On day 14, media and tissue extracts were prepared as described in Example 1, and the interferon levels in these extracts were determined using the solid phase sandwich immunoassay as described above. Table 3 gives the number of clonal duckweed lines assayed and the mean media interferon concentration for each expression construct. Table 4 shows the interferon levels within the duckweed tissue for the duckweed lines transformed with the interferon expression constructs that did not contain a signal peptide (IFN01, IFN10, and IFN12). Both the mean interferon level for all clonal lines assayed for the designated construct, and the interferon level for the top-expressing line are shown.

Table 3

Expression Construct	# of Clonal Lines Tested	Mean Interferon Concentration (ng/ml)
IFN01	41	0
IFN02	75	2.3
IFN03	41	0.18
IFN05	44	2.5
IFN07	41	1.5
IFN08	41	1.4
IFN09	41	30.3
IFN10	41	0
IFN11	39	9.9
IFN12	41	0

Table 4

IFN Construct	Mean Value % of total soluble protein	Top Expresser % of total soluble protein
IFN01	0.00003	0.0001
IFN10	0.00064	0.0074
IFN12	0.00014	0.001

The biological activity of the interferon produced by these transformed duckweed lines was assayed by the cytopathic effect inhibition assay described above. Table 5 gives the results for the top expressing line for each construct. The interferon activity is shown for the media for those constructs containing a signal peptide and the tissue for those constructs lacking a signal peptide.

Table 5

IFN Construct	Top Expresser	
	Source material	IU/ml (media) IU/mg total protein (tissue)
IFN01	Tissue	40
IFN02	Media	16,000
IFN03	Media	320
IFN05	Media	6,400
IFN07	Media	6,000
IFN08	Media	3,200
IFN09	Media	200,000
IFN10	Tissue	19,300
IFN11	Media	30,000
IFN12	Tissue	150

Example 4

A study was performed to determine the levels of IFN expressed from transgenic duckweed grown at bioproduction scales. Transgenic duckweed plants were generated using IFN expression constructs IFN02, IFN05, IFN09, IFN10, and IFN53 (see Table 2).

A minimum of 40 independent transgenic lines was screened for each of these constructs. The lines producing the highest levels of IFN expression were

analyzed further. The concentration of IFN in the media and tissue was determined by ELISA as described elsewhere herein.

Table 6 summarizes the IFN expression on both research and bioproduction scales for the constructs described above. Because *Lemna* is unique in that it grows in a very dilute inorganic media with a low protein content, the expression values in this Table are defined by the pre-purification titer. In the case of IFN53, IFN represents over 30% of the total media proteins.

Table 6: Expression of IFN in Lemna

Expression construct	Media IFN concentration for Top Expressing Line (mg/L)[c]			Mean Average Concentration for 2 Week Screening Trials (determined by ELISA)[b]	
	Research Scale (2 weeks)	Research Scale (3 weeks)	Bio-production Scale	Media (mg/L)[c]	Tissue (mg/kg tissue)[a]
IFN02	2.0	-	-	0.12	23.3
IFN05	1.1	-	-	0.13	86.7
IFN09	24.8	60	30	1.51	164
IFN10	<0.1	-	-	<0.0001	99.3
IFN53	100	300	500	15.3[d]	-

[a]Based on 1 g of tissue yielding 20 mg of protein.

[b]Based on recovery of 10 ml of media and 1 go of tissue per screening trial.

[c]Expressed as a pre-purification titer.

[d]1 week screening trial.

The antiviral activity of the duckweed-produced IFN was determined as follows. HuH7 cells were incubated for 24 hours at 37°C with 1,000 IU/ml of unpurified IFN from the duckweed media or Intron® A (Schering) as a control. The IFN was then removed and the cells were washed twice. The cells were subsequently infected with an RNA virus selected from Encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), or Sindbis at a multiplicity of infection of 1.0 for 1 hour, at which time the virus innoculum was removed. The cells were then washed three times and allowed to grow for 24 hours at 37°C. The cells were harvested, lysed by six freeze/thaw cycles, and then cell debris was removed by centrifugation. Serial dilutions of the virus were then assayed for their

cytopathic effect on monkey CV1 Vero cells. The duckweed-produced IFN exhibited similar antiviral activity to that observed for Intron®A.

The antiproliferative activity of the duckweed-produced IFN was determined as follows. Interferon-sensitive Daudi cells were seeded in microtiter plates at an initial concentration of 50,000 cells/ml. The culture were then either left untreated or were treated with 1000 IU of unpurified duckweed-produced IFN, Intron® A, or an equivalent volume of control media derived from non-transgenic plants grown under the same conditions as for the transgenic plants. After four days, the number of viable and dead cells were determined by the trypan blue-exclusion viability test.

Example 5

The sequence of the biologically active α-2b-interferon produced in duckweed was determined by mass spectrometry. The duckweed-produced interferon consisted of a mixture of five different species, with carboxy-terminus truncations of 4-8 amino acids in comparison with wild type human α-2b interferon. No full-length α-2b interferon was detected. The sequences of the variant interferons produced in duckweed are shown in SEQ ID NOS: 6-10. The predominant species produced was the 158 amino acid polypeptide shown in SEQ ID NO:9. The sequences of the corresponding precursor interferon polypeptides containing the human α-2b-interferon signal peptide are shown in SEQ ID NOS:1-5. Although the present invention is not limited to any particular mechanism, it is believed that the interferon variants were produced in duckweed by the action of an endogenous plant protease. It is the novel finding of the present invention that truncated interferon variants having the amino acid sequences shown in SEQ ID NOS:6-10 are biologically active, as shown in the Examples above.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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